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Serial Analysis of Hepatitis B Virus Core Nucleotide Sequence of Patients With Acute Exacerbation During Chronic Infection

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Recent studies suggest that hepatitis B virus (HBV) core region could be an immunological target and that amino acid (aa) substitutions are mostly restricted to a small segment located in the middle of the core region. We sequenced the middle portion of HBV core gene during the course of acute exacerbation of chronic hepatitis B, and compared aa variations between the region including ideal HLA-A2 binding motifs and the nonbinding region. Five HBeAg+ chronic hepatitis patients with subtype *adr* (three with HLA-A2 and two without HLA-A2) were selected and using polymerase chain reaction (PCR) and cloning system, the central part of core region (nt 2063 to 2365, 303 bp) was sequenced in sera from each patient at three time points; before, at the peak of, and after exacerbation of hepatitis. The second set of sera showed higher aa substitution rates in five and in three out of five patients compared with those of the first and third sera, respectively. No significant difference was found in the aa substitution rates for the region with ideal HLA-A2 binding motifs between patients with and without HLA-A2. In asymptomatic HBV carriers with persistently normal aminotransferase values, alterations of the aa sequence were not observed within the same time frame. The results suggest that aa substitutions often occur at some particular positions in the middle of HBV core region during acute exacerbation of the disease under possible host immune pressures. Furthermore, unidentified epitopes appear to exist in the central part of HBV core region and HLA-unrestricted lymphocytes may play a role in the immune response of chronic HBV carriers.

KEY WORDS: HBV core gene, amino acid divergency, HLA-A2 chronic liver disease

INTRODUCTION

Hepatitis B virus (HBV) contains a circular, partially double-stranded DNA genome of 3.2 kb [Tiollais et al., 1985]. Patients who are infected chronically with HBV have various clinical features with a wide range of liver cell damage. It is generally considered that HBV is not directly cytopathic and that immunological interaction between the host and viral antigens is responsible for the destruction of infected hepatocytes. Several studies suggest that cytotoxic T lymphocytes (CTL) play an important role for hepatocellular injury [Vento et al., 1985; Ferrari et al., 1990; Milich et al.,1989]. The polymerase chain reaction (PCR) and DNA sequencing revealed that genomic variants often occurred during the clinical course of chronic HBV infection [Lok et al., 1994; Pollicino et al., 1995].

Although the amino acid sequence of the pre-C/C region is relatively conserved compared to pre-S/S region, amino acid substitutions and deletions have been found in a small segment located in the central part of core region [Wakita et al., 1991; Takayanagi et al., 1993; Akarca and Lok, 1995]. Such changeable regions appear to be the major immunological targets of CTL in chronic HBV carriers [Ehata et al., 1992]. Therefore, such mutations may occur in the epitopes recognized by T cells. Fortuitously, recent studies revealed that the anchor motif restricted by HLA-A2 [Engelhard et al., 1993] is one of the common HLA phenotypes among Japanese. To clarify the interrelationship of core mutations and the role of HLA-A2, five patients (three with HLA-A2 and two without HLA-A2) who experienced acute exacerbation of hepatitis were investigated during a follow-up period. Their amino acid sequences of the central part of core region, in which five ideal HLA-A2 binding motifs were contained (-L------V), were determined using the sera before, at the peak of, and after exacerbation of hepatitis, and the frequency of substitutions in the HLA-

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A2 motifs between patients bearing A2 and not bearing A2 was compared.

MATERIALS AND METHODS

Patients

Serial serum samples were obtained at three different times from five patients who had acute exacerbation during the course of chronic hepatitis B. Three patients carried HLA-A2, and the other two did not. All patients were seropositive for HBsAg and HBeAg by commercial RIA kits (Abbott Laboratories, Chicago, IL). Two HBeAg-positive asymptomatic carriers (ASCs) with normal alanine aminotransferase (ALT) and two anti-HBeAg positive ASCs with normal ALT were selected as controls. For these controls, serum samples were obtained at two points within the same time frame as the former five patients. All had subtype *adr*. All samples were negative for anti-HCV antibody (Abbott HCV EIA 2nd generation kit). Quantitative assay of anti-HBc IgM levels was carried out by EIA using the AxSYM analyser (DAINABOT, Tokyo, Japan).

PCR

An aliquot of serum (100 μl) from each patient was incubated at 70°C for 3 hr in proteinase K (100 mg/ml), 0.5% (wt/vol) SDS, 5 mM EDTA, 10 mM Tris-HCl, pH 8.0. The solution was extracted with phenol-chloroform, and DNA was precipitated in the presence of carrier tRNA (10 mg/ml). A set of oligonucleotide primers was prepared, covering about a half of HBV core region [303bp, nt 2063–2365, numbered according to Okamoto et al., 1986]. These primers were synthesized using an Applied Biosystems Model 392 DNA synthesizer. The sequence of the sense primer was 5′-CTCAGGCAAGC-TATTCTGTGT-3′ (nt 2063–2083). The sequence of the anti-sense primer was 5′-GGACCTGCCTCGTCGTC-TAAC-3′ (nt 2345–2365). The PCR reaction was carried out in 100 μl of a mixture containing 10 μl of the serum DNA sample, 2.5 units of Taq DNA polymerase (Perkin-Elmer-Cetus, Norwalk, CT), 50 mM of each dNTP, and 20 pmol of each primer. The reaction was carried out in a Thermal Cycler (Perkin-Elmer-Cetus) for a total of 30 cycles according to the following protocol: denaturation at 96°C for 30 sec, annealing at 55°C for 15 sec, and extension at 72°C for 40 sec.

Cloning and Sequencing of Amplified HBV DNA

The PCR products were digested and ligated with plasmid pCR™II (Invitrogen, San Diego, CA). After ligation reaction, the recombinant plasmids were introduced into *Escherichia coli*, TA One Shot™ Competent Cell (Invitrogen), and cloned. Fluorescence-based sequencing was carried out in both directions using a 373A DNA Sequencing System and the Taq Sequencing Kit from Applied Biosystems, Inc. (Foster City, CA).

Analysis of Sequence Data

The sequenced data were analyzed by 373A Analysis Software (Applied Biosystems). Computer-assisted homology search was conducted by GENETYX Ver. 6.00 (Software Development, Tokyo, Japan).

To evaluate the frequency of aa substitutions in the individual serum sample, the number of aa residues different from the reported sequence of wild type HBV in subtype *adr* [Ono et al., 1983] were counted for each clone and the mean value in the respective five clones sequenced from the individual sample were calculated as the substitution rate [Takayanagi et al., 1993].

Statistical Analysis

Results were expressed as the mean plus minus one standard deviation and analyzed using paired and unpaired Student's t-test. *P* values of less than 0.05 were regarded as statistically significant.

RESULTS

Clinical Characteristics of the Patients

The clinical characteristics of the five patients with chronic hepatitis and serum ALT levels are summarized in Figure 1. All patients had acute exacerbation of their disease during follow-up and treatments such as IFN were not given before serum sampling. Throughout the exacerbation of each patient, HBeAg remained seropositive. Anti-HBc IgM levels rose during the exacerbation of hepatitis in three patients (cases 2, 3, and 5). In case 4, anti-HBc IgM level elevated after an episode of exacerbation of her hepatitis. The amount of HBV DNA varied widely and differed from each patient. However, the peak of HBV burden preceded that of ALT values (data not shown).

PCR Amplification and Cloning of HBV DNA

PCR products from case 1 and case 5 were visible with ethidium bromide staining after agarose gel electrophoresis as a single DNA band at the expected sizes (303 bp), respectively. Additional faint short DNA bands were observed in the PCR products from the sera obtained at the peak of exacerbation of hepatitis in cases 2, 3 and 4. These short bands were not observed in any of the control asymptomatic carriers. Five or six clones were independently selected from each serum of the individual patients and sequenced, respectively. All clones showed a high homology with the reported sequence of wild type HBV of subtype *adr* (96.2–98.8%) [Ono et al., 1983].

Clonal Analysis of HBV Core Region

Figure 2 illustrates amino acid sequences deduced from nucleotide sequences determined in this study. In cases 3 and 4, sequences of deletion mutants were also incorporated into the figure; the deletions were mapped in the middle portion of core region irrespective of ideal HLA-A2 binding motifs. Deletion mutants in case 2 were not sequenced.

Study on four asymptomatic carriers with normal ALT revealed that aa variations common to all the clones seen in one anti-HBe positive patient did not change in serial samples, and no aa substitution was found in any sample in the remaining three subjects (Fig. 3).

The mean aa substitution rate (SR) in the respective five or six clones obtained from each serum is summarized in Table I. In all 5 patients, the aa SR increased

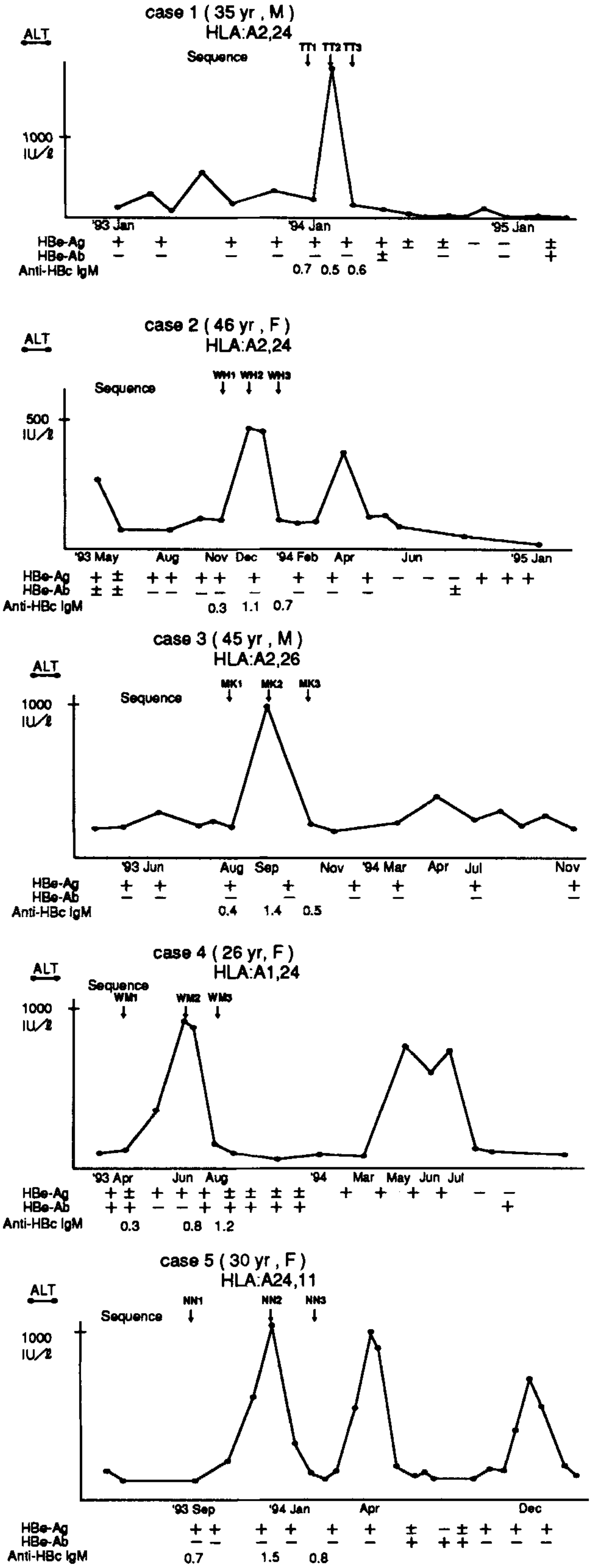

Fig. 1. The clinical course of five patients with chronic hepatitis B. They all had acute exacerbation during their disease courses. The individual sera indicated with arrows were selected for analysis. No therapeutics, such as IFN, were given before the samplings. Anti-HBc IgM levels are shown as index values by EIA technique (1.2< : positive).

at the peak of the illness, although the mean SR did not reach a significant difference compared with that of the first sample (2.44 ± 2.47% vs. 1.43 ± 1.65%). After ALT levels subsided, the SR tended to decrease again in three patients (mean SR of five patients; 1.75 ± 1.68%).

Next the aa diversities were evaluated in the area included in the five ideal binding motifs (46 aa) of HLA-A2 and in the nonbinding area (41 aa), separately, No significant difference of SR was observed between patients with HLA-A2 (1.74 ± 1.82%) and without A2 (2.07 ± 2.49%). In general, the mean SR in the nonbinding motif area was larger than that in the A2-binding motif area in both patients with A2 and without A2 throughout the study.

The amino acid substitutions were brought about at 26 different codons. Of the 26 positions, substitutions at 6 different positions (S to Y or F at 81, I to L or T at 97, S to P or Y at 106, R to K at 112, P to T or S at 130, T to A or K at 142) were seen in several patients. Among them, substitutions at codons 81 and 97 were noted in three patients. Interestingly substitution at 130 was seen in all five patients. Substitution at anchor amino acid of ideal HLA-A2 binding motif was observed in only one patient (clone TT2-2 in case 1, V to G at codon 115). In case 4, drastic clonal changes were seen in the course of the exacerbation of her hepatitis, and G at codon 63 was perfectly replaced by V.

DISCUSSION

The HBV core region, particularly the middle portion of the region, appears to be major immunological targets of CTL [Vento et al., 1985; Ferrari et al., 1990; Milich et al., 1989]. In addition, the ideal binding motifs of HLA-A2 have been defined [Engelhard et al., 1993], which is one of the common HLA phenotypes among Japanese. Thus, to confirm whether the acute exacerbations in chronic HBV carriers bearing HLA-A2 are associated with the changes in the aa sequences of the predicted binding motifs, the sequence of HBV core region was examined at three serial time points.

If the diversity of HBV genome is increased along with the elevation of HBV load just before an exacerbation of hepatitis, it is possible that some populations with aa substitutions in core gene are recognized by CTL, resulting in the killing of infected hepatocytes. In our data, SR just before the rise of ALT tended to be small compared with that at the peak of ALT. In some patients, increase in SR at the peak of their exacerbation was transient, although the clonal populations in the third sera was still high in the remaining patients. In other words, a few mutations occurred through an exacerbation of hepatitis.

In assessing aa substitutions, however, the possibility of reverse transcriptase error during HBV replication has to be taken into consideration. To clarify this issue, two HBeAg-positive and two anti-HBe-positive asymptomatic carriers with normal ALT were examined. The results showed that no aa changes were detectable among the serial samples, including one case in whom uniform aa variations were observed in all clones sequenced.

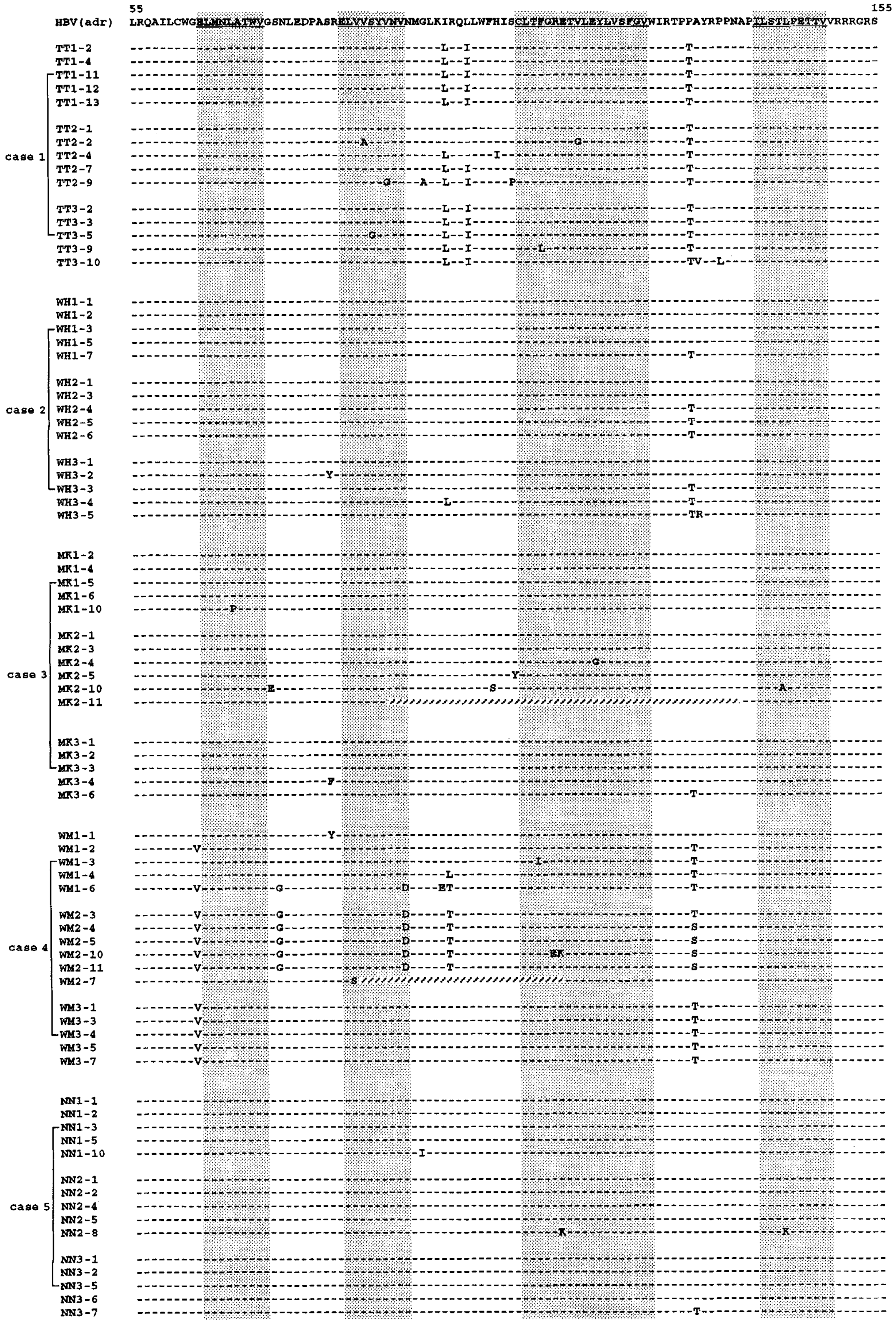
55
155
HBV(adr)
LRQAILCWGELMNLATWVGSNLEDPASRELVVSYVNVNMGLKIRQLLWFHISCLTFGRETVLEYLVSFGVWIRTPPAYRPPNAPILSTLPETTVVRRRGRS
case 1
TT1-2
TT1-4
TT1-11
TT1-12
TT1-13
TT2-1
TT2-2
TT2-4
TT2-7
TT2-9
TT3-2
TT3-3
TT3-5
TT3-9
TT3-10
case 2
WH1-1
WH1-2
WH1-3
WH1-5
WH1-7
WH2-1
WH2-3
WH2-4
WH2-5
WH2-6
WH3-1
WH3-2
WH3-3
WH3-4
WH3-5
case 3
MK1-2
MK1-4
MK1-5
MK1-6
MK1-10
MK2-1
MK2-3
MK2-4
MK2-5
MK2-10
MK2-11
MK3-1
MK3-2
MK3-3
MK3-4
MK3-6
case 4
WM1-1
WM1-2
WM1-3
WM1-4
WM1-6
WM2-3
WM2-4
WM2-5
WM2-10
WM2-11
WM2-7
WM3-1
WM3-3
WM3-4
WM3-5
WM3-7
case 5
NN1-1
NN1-2
NN1-3
NN1-5
NN1-10
NN2-1
NN2-2
NN2-4
NN2-5
NN2-8
NN3-1
NN3-2
NN3-5
NN3-6
NN3-7

Figure 2.

```
          55                                                                                                  155
HBV(adr)  LRQAILCWGELMNLATWVGSNLEDPASRELVVSYVNVNMGLKIRQLLWFHISCLTFGRETVLEYLVSFGVWIRTPPAYRPPNAPILSTLPETTVVRRRGRS

e-Ag+ASC
AI1-1     -----------------------------------------------------------------------------------------------------
AI1-2     -----------------------------------------------------------------------------------------------------
AI1-3     -----------------------------------------------------------------------------------------------------
AI1-4     -----------------------------------------------------------------------------------------------------
AI1-5     -----------------------------------------------------------------------------------------------------
case 6
44yr,M
HLA:A24,26
AI2-11    -----------------------------------------------------------------------------------------------------
AI2-12    -----------------------------------------------------------------------------------------------------
AI2-13    -----------------------------------------------------------------------------------------------------
AI2-14    -----------------------------------------------------------------------------------------------------
AI2-15    -----------------------------------------------------------------------------------------------------

KH1-1     -----------------------------------------------------------------------------------------------------
KH1-2     -----------------------------------------------------------------------------------------------------
KH1-3     -----------------------------------------------------------------------------------------------------
KH1-4     -----------------------------------------------------------------------------------------------------
KH1-5     -----------------------------------------------------------------------------------------------------
case 7
37yr,F
HLA:A2,26
KH2-11    -----------------------------------------------------------------------------------------------------
KH2-12    -----------------------------------------------------------------------------------------------------
KH2-13    -----------------------------------------------------------------------------------------------------
KH2-14    -----------------------------------------------------------------------------------------------------
KH2-15    -----------------------------------------------------------------------------------------------------

e-Ab+ASC
NS1-1     --------V---T------N--------D---N---T----------------------------------------------------------------
NS1-2     --------V---T------N--------D---N---T----------------------------------------------------------------
NS1-3     --------V---T------N--------D---N---T----------------------------------------------------------------
NS1-8     --------V---T------N--------D---N---T----------------------------------------------------------------
NS1-9     --------V---T------N--------D---N---T----------------------------------------------------------------
case 8
32yr,M
HLA:A2,24
NS2-1     --------V---T------N--------D---N---T----------------------------------------------------------------
NS2-2     --------V---T------N--------D---N---T----------------------------------------------------------------
NS2-3     --------V---T------N--------D---N---T----------------------------------------------------------------
NS2-4     --------V---T------N--------D---N---T----------------------------------------------------------------
NS2-5     --------V---T------N--------D---N---T----------------------------------------------------------------

FM1-1     -----------------------------------------------------------------------------------------------------
FM1-3     -----------------------------------------------------------------------------------------------------
FM1-4     -----------------------------------------------------------------------------------------------------
FM1-5     -----------------------------------------------------------------------------------------------------
FM1-6     -----------------------------------------------------------------------------------------------------
case 9
39yr,F
HLA:A24,31
FM2-2     -----------------------------------------------------------------------------------------------------
FM2-4     -----------------------------------------------------------------------------------------------------
FM2-5     -----------------------------------------------------------------------------------------------------
FM2-6     -----------------------------------------------------------------------------------------------------
FM2-7     -----------------------------------------------------------------------------------------------------
```

Fig. 3. Deduced amino acid sequences of central part of HBV core region in four asymptomatic HBV carriers with normal ALT values. Cases 6 and 7 are seropositive for HBeAg, while cases 7 and 8 are anti-HBeAg-positive. The amino acid sequences at two different points are serially shown. The second sample had an interval of five months after the first sampling point in each patient.

As for deletions in HBV core region, these were found only in the sera obtained at the peak of exacerbation of hepatitis, but not in asymptomatic carriers. These data support the possibility that mutations and deletions in the HBV core gene may occur under strong immune pressure such as acute exacerbation of hepatitis. In fact, case 4 had drastic aa substitutions as a result of the exacerbation of her illness. In this case, G at codon 63 was replaced by V after the peak of ALT. Such substitutions may be regarded as a result of escape from immune pressures [Pircher et al., 1990; Chuang et al., 1993]. Carman et al. [1994] reported that mutations within the preC/C region are related to disease severity in both anti-HBe-positive and HBeAg-positive patients with active liver disease. These studies are consistent with our hypothesis that mutations within core region would result from immune pressure under increasing severity of hepatitis.

Only a single aa substitution will affect CTL response by changing peptides' binding affinity to major histocompatibility complex (MHC) class I or changing efficiency of recognition by T cell receptor (TCR), and such mutants may act as natural TCR antagonists [Bertoletti et al., 1994]. But in the present study, mutations within an ideal HLA-A2 binding motif occurred at a low frequency and no significant difference was found between

Fig. 2. Deduced amino acid sequences of central part of HBV core region in five chronic hepatitis patients. Only residues different from the reported sequence of wild type HBV in the subtype *adr* are indicated. The amino acid sequences at three different points (before, at the peak of, and after the exacerbation of hepatitis) are serially shown. HLA-A2 binding motifs are highlighted in darkened areas. (////) indicates deletions.

TABLE I. Substitution Rate of Amino Acids in a Part of HBV Core Gene in Patients With Chronic Hepatitis B

Patient no.	Blood samples	Substitution rate of amino acid sequence		
		aa (%)	in A2 (%)[a]	in $\overline{A2}$ (%)
1	TT1	3.45 ± 0	0	3.45 ± 0
	TT2	3.68 ± 2.06	0.69 ± 1.03	2.99 ± 1.92
	TT3	4.37 ± 0.96	0.46 ± 0.63	3.91 ± 1.02
2	WH1	0.23 ± 0.52	0	0.23 ± 0.52
	WH2	0.69 ± 0.63	0	0.69 ± 0.63
	WH3	1.38 ± 0.97	0	1.38 ± 0.97
3	MK1	0.23 ± 0.52	0.23 ± 0.52	0
	MK2	1.15 ± 1.41	0.46 ± 0.63	0.69 ± 1.02
	MK3	0.46 ± 0.63	0	0.46 ± 0.63
Mean of 1, 2, 3		1.74 ± 1.82	0.20 ± 0.19	1.53 ± 1.67
4	WM1	2.99 ± 2.24	0.46 ± 0.63	2.53 ± 1.89
	WM2	6.21 ± 1.02	1.61 ± 1.02	4.60 ± 0
	WM3	2.30 ± 0	0	2.30 ± 0
5	NN1	0.23 ± 0.52	0	0.23 ± 0.52
	NN2	0.46 ± 1.02	0.46 ± 1.02	0
	NN3	0.23 ± 0.52	0	0.23 ± 0.52
Mean of 4, 5		2.07 ± 2.49	0.42 ± 0.38	1.65 ± 2.11

[a]A2, sequenced region with HLA-A2 binding motifs; $\overline{A2}$, sequenced region outside of HLA-A2 binding motifs. Results were expressed as mean ± S.D. (%) of substitution relative to the *adr* subtype in the clones obtained from each sera.

patients bearing HLA-A2 and not bearing A2. In addition, mutation in an anchor position was observed only in one clone in case 1 (codon 115, V to A) among three patients with HLA-A2. The latter finding raises the possibility that these mutants, although they exist as a minor population, may act as a TCR antagonist and evade CTL reaction. In our study, we analyzed only a part of HBV core gene and thus it is likely that CTL restricted to other region could still participate in an immune response.

The anti-HBc IgM levels were examined serially during the exacerbation of hepatitis. Sjogren and Hoofnagle [1985] reported that the presence of anti-HBc IgM in chronic HBV carriers indicated active immune response to viral replication. It is interesting to note that anti-HBc IgM levels increased during the exacerbation of hepatitis in three out of five cases.

In the central part of the HBV core region, clustering of aa mutations and deletions were reported [Wakita et al., 1991; Ehata et al., 1992; Ackrill et al., 1993]. Within that region, many epitopes for B cell, CD4+ T cell and CD8+ T cell are found [Salfeld et al., 1989; Colucci et al., 1988; Ferrari et al., 1991; Bertoletti et al., 1991, 1993; Penna et al., 1991; Missale et al., 1993]. Therefore, aa substitutions within that region might affect the immunological events of the host such as expression and/or localization of core antigen as well as the immune *response* by lymphocytes [Liao and Ou, 1995; Tordjeman et al., 1993; Carman et al., 1995].

In summary, among the patients bearing HLA-A2, no remarkable or specific aa substitutions were observed within five ideal HLA-A2 binding motifs throughout an exacerbation of hepatitis. These results suggest two possibilities. One is that unidentified epitopes may exist in the central part of HBV core region. Another is that lymphocytes including CTL, not restricted by HLA class I [Maccario et al., 1995], may play a role in the immune response. The meaning of mutations clustering in the central part of HBV core region awaits further investigation with large-scale patients, in which cellular and humoral immune systems are taken into consideration.

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